LETTER TO JMG

Polyalanine and polyserine frameshift products in Huntington's disease

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Codon reiteration disorders are caused by abnormal expansions of either polyglutamine or polyalanine tracts within the coding region of a protein. These mutations impair normal protein folding, resulting in aggregate formation in the affected tissues. Huntington's disease is the most common of the nine disorders caused by polyglutamine expansion mutations. The most extensively studied polyalanine expansion disorder is oculopharyngeal muscular dystrophy. There may be a link between diseases caused by polyglutamine and polyalanine expansion mutations as it has been shown that the expanded CAG/polyglutamine tract within the *SCA3* gene can shift to the CGA/polyalanine frame. Here, we show that this frameshifting phenomenon is more widespread and occurs in Huntington's disease. We have shown both +1 frameshift and +2 frameshift products (which may contain polyalanine or polyserine tracts, respectively) in human postmortem Huntington's disease brains and in a transgenic mouse model of Huntington's disease. Our data suggest that +1 and +2 frameshift products are generated at low levels. This may be relevant to the pathogenesis of human Huntington's disease, as we have previously shown that both polyserine and polyalanine-containing proteins are modifiers of mutant huntingtin toxicity, with low expression levels of polyalanine-containing proteins having a protective effect.

Huntington's disease is an autosomal dominant neurodegenerative condition caused by the abnormal expansion of a (CAG) n repeat (n>35) encoding a polyglutamine tract within the N terminus of huntingtin.[1] The resultant mutant huntingtin forms intraneuronal aggregates, which are the pathological hallmark of the disease.[2] Huntington's disease is one of the nine codon reiteration disorders caused by polyglutamine expansion mutations that include spino-cerebellar ataxia (SCA) 1, 2, 6, 7 and 3, spinobulbar muscular atrophy and dentatorubal-pallidoluysian atrophy.[3] Another class of codon reiteration disorders exists—namely, those caused by polyalanine expansion mutations.[4] The most extensively studied of these is oculopharyngeal muscular dystrophy (OPMD),[5] in which the expansion mutation also leads to aggregation of the mutant protein in the affected tissue.[6 7]

There may be a link between diseases caused by polyglutamine and polyalanine expansion mutations, as frame shifting of the original *SCA3* gene product encoding CAG/polyglutamines to GCA/polyalanines has been shown.[8] Ribosomal slippage during translation of the SCA3 protein has been proposed as the mechanism resulting in shifting from the polyglutamine to the polyalanine-encoding frame.[9] It is unclear how widespread this frameshifting phenomenon is, and whether it occurs in polyglutamine expansion disorders other than SCA3. We decided to look for +1 and +2 frameshift products in the most common polyglutamine expansion disorder, Huntington's disease. This is especially important, as we have recently shown that polyalanine and polyserine-containing proteins are modifiers of mutant huntingtin toxicity.[10] We have generated antisera to epitopes at the C terminus of huntingtin exon 1 (Htt exon 1) that would be predicted if frame shifting occurred upstream in the protein. Using these frameshift antibodies, we showed the presence of both +1 and +2 frameshift products in Huntington's disease during postmortem examination of brains and transgenic mouse brain samples.

METHODS

Antibody production

Antibodies were made commercially (Sigma-Genosys, Suffolk, UK) by inoculating rabbits with peptides corresponding to the predicted C terminus of frameshift huntingtin exon 1 (APAAAPAATRPGCG, Htt^{+1}; SRPRRRPRRHPARLW, Htt^{+2}) conjugated to keyhole limpet haemocyanin (KLH). Peptide antigen and preimmune serum from the rabbit before inoculation were used in control experiments. To test whether the antibody was directly able to detect its antigen, the antigen (1–5 μg) was spotted on nitrocellulose membrane (Hybond ECL membrane; Amersham Biosciences, GE Healthcare, Bucks, UK), which was subsequently processed as a western blot. Membranes were incubated with antibody (diluted in 5% dried milk in 0.1 M phosphate-buffered saline, 0.1% Tween-20, pH 7.6), followed by horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences; 1:5000), and signal visualised using enhanced chemiluminescence reagent (ECL; Amersham Biosciences) and by exposing the membrane to ECL Hyperfilm (Amersham Biosciences).

Immunohistochemistry

Immunohistochemical examination of paraffin wax-embedded sections of caudate or putamen from grade III Huntington's disease and control brains was carried out using standard peroxidase labelling with an Avidin Biotinylated enzyme Complex (ABC) kit (Vector Labs, Peterborough, UK). All human samples were studied with approval from the local ethics committee.

Sections from two brains with Huntington's disease and two control brains were used in all experiments. Peroxidase labelling of free-floating mouse brain sections from transgenic (Ross/Borchelt, HD-N171-N82Q[11]) mice or non-transgenic littermates was carried out using a standard protocol. For immunofluorescence labelling, free-floating sections were incubated with fluorophore-conjugated secondary antibodies (Alexa Fluor antibodies; 1:500; Invitrogen, Paisley, UK) after primary antibody labelling. Primary antibodies used included anti-huntingtin (EM48; Millipore, UK, 1:200) and anti-ubiquitin (Dako, Cambridgeshire, UK, 1:2000). Frameshift antibodies and preimmune serum were used at a dilution of 1:500. For pre-absorption control, frameshift

antibodies were incubated overnight at 4°C, with the relevant peptide antigen at 50 mg/ml. Controls, including primary antibody omitted and primary antibody alone in double-labelling experiments, were carried out in parallel. Three sections of transgenic mouse cortex with Huntington's disease were stained using anti-Htt+1 or anti-Htt+2 antibody and about 200 cells with aggregates were scored per section.

RESULTS

To test whether frameshift products occurred in brains with Huntington's disease, we raised polyclonal antibodies to synthetic peptides corresponding to novel epitopes predicted to be at the C terminus of huntingtin exon 1 after putative +1 or +2 frame shifts (fig 1A). A dinucleotide deletion or single-nucleotide insertion within the polyglutamine tract of huntingtin exon 1 would shift the CAG, polyglutamine-encoding frame by +1 (+1 frame shift) to the GCA, polyalanine-encoding frame and introduce a novel epitope to the C terminus of Htt exon 1 (APAAAPAATRPGCG). Likewise, a single-nucleotide deletion or a dinucleotide insertion would shift the frame by +2 (+2 frame shift), convert the CAG repeat into a AGC repeat encoding a polyserine tract, and introduce a novel epitope to the C terminus of Htt exon 1 (SRPRRRPRRHPARLW). Two independent polyclonal antibodies were raised against each epitope (Htt+1 antibody 1, Htt+1 antibody 2, Htt+2 antibody 1 and Htt+2 antibody 2) by inoculating two separate rabbits with each peptide antigen.

To determine the specificity of the Htt+1 and Htt+2 antibodies, antigen was spotted on nitrocellulose, which was subsequently incubated with antibody and processed as a western blot. Both Htt+1 antibodies recognised the +1 (polyalanine-shifted) antigen when spotted on nitrocellulose, but did not recognise +2 (polyserine-shifted) antigen. Likewise, the Htt+2 antibodies recognised +2 antigen when spotted on nitrocellulose, but not +1 antigen. Neither +1 nor +2 antigens were recognised by preimmune serum (fig 1B.). We saw no additional bands on western blots of brain lysates of wild-type mice when probed with anti-Htt+1 or anti-Htt+2 , compared with preimmune serum, confirming that the antibodies do not recognise abundant proteins in normal brains (data not shown).

Immunohistochemistry using Htt+1 antibody 1, Htt+1 antibody 2, Htt+2 antibody 1 and Htt+2 antibody 2 showed the presence of Htt+1 and Htt+2 frameshift products in intranuclear aggregates within the caudate/putamen of brains with grade III Huntington's disease but not in age-matched control brains (fig 2A). No labelling was detected when brains with Huntington's disease were incubated with preimmune serum or antibody that had been preabsorbed with its antigen. In addition, no Htt+1 or Htt+2 immunoreactivity was observed in areas of human brains with SCA2 and SCA7, where we have previously shown abundant aggregates,[12] further confirming the specificity of the antibodies (data not shown).

Both frameshift products were also detected in the cortex of transgenic mice with Huntington's disease[11] using DAB immunohistochemistry, but not in non-transgenic littermates (fig 2B). Again, no immunoreactivity was seen when the samples were incubated with preimmune sera or with preabsorbed antibody (data not shown). Using immunofluorescent double labelling, we found that about 4% of the EM48 (huntingtin N-terminal antibody) labelled inclusions were also stained by frameshift antisera in the cortex of transgenic mouse brains with Huntington's disease (fig 2C).

No products were detected on western blots of HD transgenic mouse brains (data not shown). This is not unexpected, given the low proportions of frameshifted products detected immunohistochemically and the likelihood

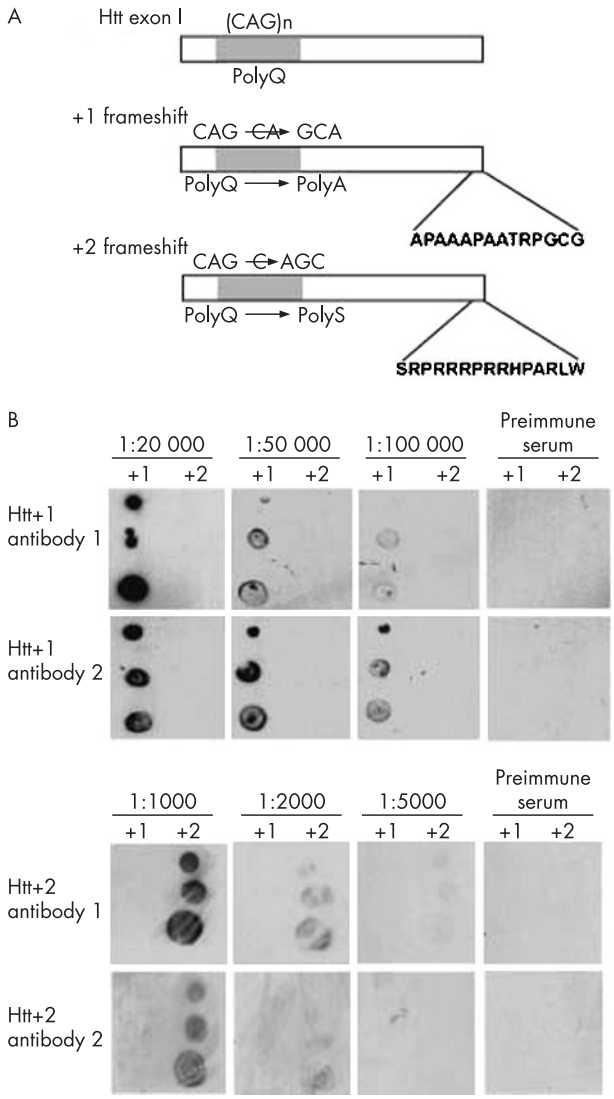

Figure 1 Generation and specificity of antibodies raised against frameshift huntingtin (Htt+ and Htt+2). (A) Schematic representation of huntingtin exon 1 (Htt exon 1) and the production of frameshift proteins. A dinucleotide deletion or a single-nucleotide insertion in the trinucleotide repeat tract would shift the frame by +1 (+1 frame shift), convert the CAG repeat that encodes a polyglutamine tract into a GCA repeat encoding a polyalanine tract and introduce a novel epitope to the C terminus of Htt exon 1 (APAAAPAATRPGCG). Likewise, a single-nucleotide deletion or a dinucleotide insertion would shift the frame by +2 (+2 frame shift), convert the CAG repeat into a AGC repeat encoding a polyserine tract and introduce a novel epitope to the C-terminus of Htt exon 1 (SRPRRRPRRHPARLW). Polyclonal antibodies were raised to synthetic peptides corresponding to the novel epitopes. (B) Synthetic peptide antigens (+1 and +2; see fig 1A) used to generate antibodies against frameshift huntingtin were spotted on nitrocellulose (5, 2 and 1 μl), which was probed with Htt+1 and Htt+2 antibodies at varying dilutions (1:1000, 1:2000, 1:5000, 1:10 000, 1:20 000, 1:50 000 or 1:100 000) from two different animals (antibody 1, antibody 2) or with preimmune serum from the same animal. Htt+1 antibody 1 and Htt+2 antibody 1 were used for subsequent experiments.

that the frame shift may occur at multiple points along the CAG/polyglutamine tract (in accordance with what was previously seen in SCA3[9]). This would result in a ladder of multiple products, precluding concentration of the low-abundance frame shifts into a single band. Frameshifted huntingtin was also not detected (by immunocytochemistry) in stable, inducible PC12 cell line:[13] even after 1 month's expression of an Htt exon 1 Q74 fragment—the construct

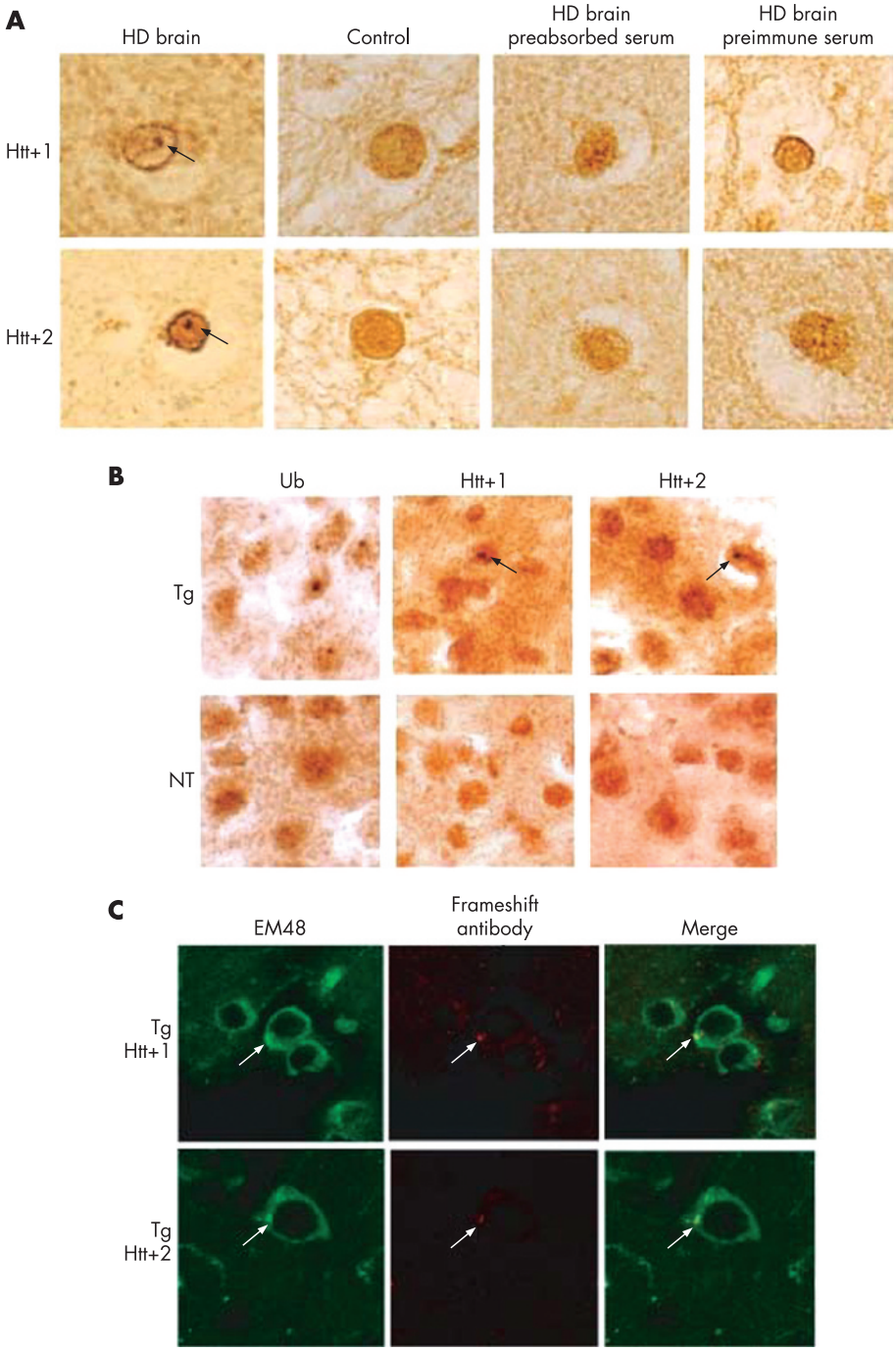

Figure 2 Frameshifted huntingtin in postmortem human and transgenic brains. (A) Brains with grade III Huntington's disease were stained with the Htt+1 and Htt+2 antibodies described in fig 1. Frameshift products Htt+1 and Htt+2 are present in intranuclear aggregates (arrow) within the caudate/putamen of brain samples with Huntington's disease (Huntington's disease brain). No immunoreactivity was seen in brains of age-matched controls (control) and those with grade III Huntington's disease at postmortem examination, incubated with preimmune serum (Huntington's disease brain preimmune serum) or with antibody that had been preabsorbed with 50 mg/ml of its peptide antigen (Huntington's disease brain preabsorbed serum). (B) Sections from late-stage (about 22 weeks old) Huntington's disease transgenic mouse brains[11] were labelled by diaminobenzidine immunohistochemistry using the antibodies described in fig 1A. Antibodies against Htt+1 and Htt+2 and ubiquitin (Ub) label aggregates (arrow) within the cortex of transgenic (Tg) mice but not in non-transgenic (NT) littermates. (C) Fluorescent double labelling of transgenic mouse brain sections (Tg) shows co-localisation of frameshift huntingtin (red), with aggregates labelled using the huntingtin EM48 antibody (green).

expressed in these cells is long enough to potentially allow formation of Htt+1 and Htt+2 neo-epitopes and after 1 month of expression, 95–100% of cells contained aggregates. This suggests that frame shifting of huntingtin is a time-dependent event that occurs at low levels.

DISCUSSION

We have shown the presence of +1 and +2 frameshift products in human, Huntingdon's disease postmortem brains and in brain samples from a transgenic mouse model of Huntington's disease. We raised antibodies against epitopes that would be expected to occur in the C terminus of Htt exon 1 if +1 or +2 frame shifting occurred in the upstream polyglutamine tract. Htt+1 and Htt+2 antibodies labelled inclusions in brains with Huntington's disease at postmortem examination, but did not label age-matched control brains. Likewise, antibodies were generated against frameshift huntingtin-labelled transgenic mouse brains with Huntington's disease but not against age-matched, non-transgenic littermates. Labelling was not seen when samples were incubated with preimmune serum (taken from the rabbit before immunisation with antigen) or with antibody that had been preabsorbed with antigen. Further evidence for the specificity of our Htt+1 and Htt+2 antibodies comes from their inability to label areas of SCA2 and SCA7 brains where we have previously shown abundant aggregates.[12] In addition, Htt+1 and Htt+2 antibodies did not detect additional bands on western blots of brain lysates of wild-type mice compared with preimmune serum, confirming that the antibodies do not recognise abundant proteins in normal brains. We acknowledge that the antibodies used detect frame

shifting in exon 1 that may have occurred at any codon upstream of the epitope, not necessarily within the CAG tract. Therefore, +1 and +2 frameshift products may or may not contain full-length polyalanine or polyserine tracts. However, previous data suggest that frame shifting occurs preferentially within expanded CAG tracts and is dependent on CAG length.[9]

Immunofluorescent double labelling showed colocalisation of frameshift products to EM48-labelled, huntingtin-containing aggregates. Although we have previously shown in cell culture that polyserine-containing proteins can localise to huntingtin aggregates, we also showed that polyalanine and polyglutamine-containing proteins do not colocalise.[10] The colocalisation of the +1 frameshift products with the predominant aggregate signals in vivo may occur because the EM48 antibody labels an epitope upstream of polyQ, and will also detect frameshift products. We are constrained by the use of EM48 as this antibody does not discriminate between normal and frameshift products. EM48 recognises the first 256 amino acids of huntingtin.[14] EM48-positive aggregates that are decorated by frameshift antibodies will therefore contain either frameshift product alone or frameshift product with non-frameshift product. As frameshift-positive aggregates represent only 4% of the total EM48 aggregates, frame shifts probably represent a rare species. However, we acknowledge that the number of aggregates positive for frameshift product immunoreactivity is determined by the amount of frameshift product and the ability of these products to form, or be trapped, in aggregates.

Frameshift products were found in the brains of patients with Huntington's disease and transgenic mice (putatively in association with huntingtin aggregates), but were not found in normal brains or in brains with SCA2 or SCA7 brains containing aggregates. The neo-epitopes that are detected by frameshift antibodies are not seen in SCA2 or SCA7. Despite this, our data cannot resolve whether frame shifting occurs exclusively in Huntington's disease gene alleles with expanded CAG repeat or whether it can occur in both normal and expanded Huntington's disease gene alleles, but the frameshift products are visible only when trapped in huntingtin-containing aggregates.

Previous data suggested that SCA3 could not shift +2 to the polyserine frame and that polyalanine frameshift products were generated at high levels.[8 9] Here, we show the presence of +2 frameshift products in Huntington's disease. In addition, double labelling suggested that frame shifts were present in only 4% of aggregates, consistent with low levels of +1 frameshift formation. This low level of polyalanine frameshift production is further supported by the lack of detection (by immunocytochemistry) of +1 frame shifts in a Huntington's disease exon 1 cell model[13] even after being induced to express transgene for 1 month. Together, this suggests that the formation of huntingtin frameshift products occurs at low levels and is a time-dependent process. We cannot exclude the possibility that the inability to detect frameshift products in our PC12 model may be due to the transgene promoter. This is unlikely, as we see frameshift products in two different systems with mutant huntingtin expression being driven by two distinct promoters (the endogenous huntingtin promoter in human brains with Huntington's disease and the mouse prion promoter in transgenic mice). However, it is formerly possible that expression levels (which are mostly determined by promoter type) may influence the formation of frameshift products or the ability to detect them.

As frame shifts occur in both Huntington's disease and SCA3, the most straightforward explanation is that repeat sequences are particularly susceptible to such frame shifting. Indeed, it has been previously suggested that ataxin 3 frameshift events occur more frequently within longer CAG repeats.[9] Nevertheless, we cannot rule out the possibility that perturbations induced in cells expressing constructs with expanded polyglutamine tracts may induce frame shifts in genes without repeat sequences.

Our data may have consequences for the pathogenesis of Huntington's disease as we have previously shown polyserine and polyalanine-containing proteins to be modifiers of huntingtin toxicity.[10] Low levels of polyalanine protected against polyQ-induced toxicity in cells and flies by induction of heat shock response. However, it is difficult to deal with the exact outcome of polyalanines in the pahogenesis of Huntington's disease experimentally and we cannot rule out the possibility that polyalanine frameshifts can exacerbate Huntington's disease pathology in some cells.

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